JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2005, p. 716–720 Vol. 43, No. 2
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.2.716–720.2005

Characterization of Virus Isolates by Particle-Associated Nucleic Acid PCR

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Received 2 June 2004/Returned for modification 27 August 2004/Accepted 1 October 2004

Diagnostic virus isolation is still frequently used, particularly from respiratory tract secretions. Testing positive virus cultures for all possible viruses is time-consuming, and unexpected or unknown viruses may escape detection. Therefore, a novel random PCR approach was developed that allows sequence-independent amplification of viral nucleic acids from virus isolation-positive cultures. Selectivity for viral sequences is obtained by preferential isolation of nucleic acids that are particle associated and resistant to nucleases. Using primers with a degenerated 3′ end, the isolated nucleic acids are amplified and the randomly amplified PCR products are cloned and sequenced. As proof of the concept, the PAN-PCR approach was applied to supernatants of coxsackievirus B3 and murine adenovirus type 1-infected cells. Enterovirus and adenovirus sequences were obtained, demonstrating that the random PCR approach allows detection of RNA and DNA viruses. As a first application of this PAN-PCR approach, we characterized a virus isolate from mouth-washing material of a patient with chronic fatigue syndrome and high antibody titers to coxsackievirus B2. The virus isolate had tested negative for enteroviruses and respiratory viruses (influenza viruses A and B, parainfluenza virus types 1 to 3, respiratory syncytial virus, and adenovirus) by immunofluorescence and PCR. Particle-associated, nuclease-resistant RNA and DNA were prepared from the supernatant of infected cells. The DNA and the reverse-transcribed RNA were randomly amplified, and PCR products were cloned and sequenced. Of 25 sequences obtained from the DNA preparation, 24 contained herpes simplex virus type 1 (HSV-1) sequences from 14 different loci spread over the HSV-1 genome. This result was confirmed by using a standard diagnostic HSV-PCR, demonstrating that the PAN-PCR correctly identified the virus isolate. Although the identification of HSV-1 in mouth-washing material is not surprising in retrospect, it clearly demonstrates the applicability of the PAN-PCR approach. This method should be particularly useful for characterizing virus isolates that have tested negative for all expected viruses and for identifying unknown viruses.

Recent identification of human metapneumovirus (13), the severe acute respiratory syndrome (SARS) coronavirus (4), and HCoNL63 coronavirus (5, 14) suggests that other viruses pathogenic to humans have either not yet been discovered or might newly emerge. This notion is further supported by the observation that in ca. 30 to 45% of patients with clinical signs of lower respiratory tract infections an etiological agent cannot be identified by laboratory diagnosis even if sensitive PCR methods are used (9, 10). In the recent past, one successful approach to identify novel viruses has been virus isolation. After exclusion of known viruses, the virus isolates were subsequently characterized by molecular biological approaches, leading to the identification of genomic fragments of the virus. In addition to the use of degenerate primers from highly conserved viral sequences under low-stringency conditions (4, 11) and DNA microarrays catching highly conserved viral sequences (16), differential display has been used to identify sequences unique to cultures infected with the unknown virus isolate (14). Although the first two approaches are limited by the conservation of viral genomes, the latter ones are rather time-consuming and technically demanding. Usually only one or a few fragments of viral genomes are obtained, and many viruses might escape detection. We therefore developed a more widely applicable method for characterizing unknown virus isolates. Viral nucleic acids were selectively isolated from infected cell cultures by virtue of the size and density of virus particles and nuclease resistance of viral nucleic acids within virus particles. By using a sensitive novel random PCR, viral nucleic acids were amplified in a sequence-independent manner, cloned, and characterized by sequence analysis.

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MATERIALS AND METHODS

Cell culture and virus production. HeLa, Vero, or NIH 3T3 cells cultured in Dulbecco modified Eagle medium with 5 to 10% of fetal calf serum were used to passage human adenovirus type 5 (Ad5), a patient isolate, coxsackievirus B3 (Cox-B3), and murine Ad1.

Purification of genomic human Ad5 DNA. Ad5 was purified from cell lysates by two rounds of CsCl density centrifugation (6), dialyzed against 1,500 ml of phosphate-buffered saline (PBS) with 1 mM $MgCl_2$ and 10% glycerol four times (1 h each) at 4°C, and stored at −80°C. Viral DNA was isolated with the Blood-Mini-Kit (Qiagen, Hilden, Germany).

Purification of particle-associated nucleic acids (PANs). For enrichment of viral particles from the supernatant of infected cells, 11 ml of culture supernatant was clarified (3,220 × *g*, 30 min) and subsequently filtered through a 0.22-μm-pore-size sterile filter to eliminate particles of higher density and mass such as bacteria, eukaryotic cells, or fragments of them. To concentrate virus particles and separate them from particles of lower density, 10 ml of sterile filtered supernatant were layered onto 2 ml of 30% (wt/vol) sucrose-PBS, followed by centrifugation for 3 h in an SW41 rotor at 30,000 rpm. For preparation of DNA, the pellet was resuspended in 250 μl of PBS containing 20 mM $MgCl_2$. To degrade DNA that is not inside the particle, a DNase step was performed by the addition of 5 U of DNase I, followed by incubation at 37°C for 30 min, followed

in turn by DNA extraction with the Blood-Mini-Kit (Qiagen). For RNA preparations, viral pellets were resuspended in 200 μl of PBS, followed by extraction of nucleic acids with the Blood-Mini-Kit. Nucleic acids were eluted in 50 μl of RNase-free water and digested with DNA-free DNase (Ambion, Austin, Tex.).

Purification of PANs from a patient-derived isolate. A virus isolate was recovered from the mouth-washing material of a patient with chronic fatigue syndrome by inoculation of HeLa cells. Purification of PANs was done as described above, but an additional purification step between filtration and ultracentrifugation was included. For this purpose the filtered supernatant was layered onto a sucrose step gradient. Each layer of the gradient consisted of 3 ml of sucrose in PBS with concentrations of 60, 50, 40, 30, and 20% (wt/vol [from bottom to top]). Ultracentrifugation was done in an SW28 rotor for 4 h at 25,000 rpm. Fractions (3 ml) were collected, and the pellet was resuspended in 3 ml of PBS. An aliquot of 250 μl was taken for inoculation of HeLa cells and monitoring of cytopathic effects (CPEs) after 18 h. The remaining volume of virus-positive fractions was adjusted to 11 ml with PBS, and particles were pelleted by ultracentrifugation through 1 ml of a 20% (wt/vol) sucrose-PBS bed by using an SW41 rotor at 30,000 rpm for 2 h. Pellets were resolved in 400 μl of PBS, from which 200 μl was taken for DNA and RNA isolation, respectively.

Reverse transcription. Because preliminary experiments indicated more efficient amplification of double-stranded rather than single-stranded DNA double-stranded cDNA was synthesized from RNA samples. For this purpose, we used a cDNA Synthesis System (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Then, 19 μl of purified RNA was taken for each reaction. For priming of first-strand synthesis, we used a 24-mer oligonucleotide with randomized sequence. Purification of double-stranded cDNA by phenol-chloroform extraction and ethanol precipitation was done as described previously (12) by using 10 μg of glycogen per reaction to improve precipitation efficiency. Precipitates were resolved in 20 μl of UV-treated water and used as templates in the random PCR.

Primer extension and random PCR. To avoid amplification of DNA possibly contaminating reagents used for annealing and extension of the random primer, the annealing buffer consisting of 5 pmol of primer K-random-s (GAC CAT CTA GCG ACC TCC AC*M NN MNM*), 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and the extension buffer (240 mM NaCl, 12 mM $MgCl_2$, and 0.4 mM concentrations of each deoxynucleoside triphosphate [dNTP]) were digested with 4 U of DNase I for 15 min at 37°C in a final volume of 19 and 5 μl, respectively. DNase was degraded by addition of 0.12 U of proteinase K (PCR grade; Roche) and incubation at 55°C for 30 min, followed by inactivation of proteinase K at 75°C for 20 min.

The DNase-treated annealing buffer was complemented with 1 μl of template DNA, followed by incubation at 22°C overnight. To dissolve secondary structures, the reaction was heated to 65°C for 15 min. Then, 1 U of T4 DNA polymerase and 5 μl of extension buffer were added to the reaction, and primer extension was performed at 12°C for 20 min, followed by inactivation of T4 DNA polymerase at 75°C for 10 min. Subsequently, PCR amplification of primer extension products was performed in a total reaction volume of 15 μl containing 5 μl of the reaction described above, 0.4 μM primer K-s (GAC CAT CTA GCG ACC TCC AC), 0.2 mM concentrations of each dNTP, 6.6 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 33.4 mM KCl, and 0.75 U of *Taq* DNA polymerase (Amersham Biosciences, Freiburg, Germany). Cycling was performed as follows: 1 cycle of 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Products were finally visualized on an ethidium bromide-stained agarose gel. Preliminary experiments showed that it is sometimes beneficial to digest template DNA into smaller fragments prior to amplification. So, in each case, two reactions were done with either DdeI-digested DNA or undigested DNA.

Cloning and sequencing of PAN-PCR products. Fragments resulting from a random PCR were cloned into a pCR2.1 vector by using the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany). Bacteria were cultured on ampicillin–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) agar plates, allowing blue-white screening. White colonies were picked and cultured for 1 h in 1 ml of Luria-Bertani broth plus ampicillin. For subsequent PCR amplification of the cloned inserts, 1 μl of bacterial suspension was added to the PCR containing 0.2 μM concentrations of the primers M13fwd (GTA AAA CGA CGG CCA G) and M13rev (CAG GAA ACA GCT ATG AC), 2 mM concentrations of each dNTP, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, and 1.25 U of *Taq* DNA polymerase in a total reaction volume of 25 μl. Cycling was performed as follows: 1 cycle of 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. To avoid repeated sequencing of the same cloned PCR products or primer dimers, only PCR products that were larger than 260 bp and that differed in size were identified by gel electrophoresis. After ethanol precipitation, the clonal PCR products were sequenced with the M13fwd primer at GENterprise Sequenzierservice (Mainz, Germany).

Sequences obtained were analyzed for homologies to nucleotide sequences in the GenBank database by using the BLASTn and the Vector NTI 6 (InforMax, Inc.) software. Further analysis by a translated BLAST search (BLASTx) was done for sequences that showed no or low homologies at nucleotide level.

Real-time quantitative PCR. Viral nucleic acids from crude supernatants of infected cultures were prepared by using the Blood-Mini-Kit (Qiagen) according to the manufacturer's recommendations. Quantitative detection of human adenoviral DNA was performed on a LightCycler instrument (Roche) by using a TaqMan probe (8)-based real-time PCR as described previously (7). Enterovirus RNAs and MAV-1 were quantified by in-house TaqMan-based real-time PCR assays on the Rotor-Gene RG-3000 system (Corbett Research, Mortlake, New South Wales, Australia). Quantification of HSV-1 DNA was done in a LightCycler system by means of a modified real-time PCR protocol described previously (3).

RESULTS

Sequence-independent amplification by random PCR. To be able to detect unknown viruses with high sensitivity, we started out to develop a PCR approach allowing sequence-independent amplification of viral nucleotide sequences. This approach is based on a random PCR ideally leading to representative amplification of all nucleic acid templates entered into the reaction. In this method (PAN-PCR), the specificity for viral nucleic acids is not provided by the PCR itself but by selective isolation of PANs.

A random primer was used containing a 20-nucleotide constant 5′ part and a 6-nucleotide degenerate 3′ end with the sequence MNNMNM. The constant part and the degenerate sequence are designed to avoid formation of primer dimers. After denaturation of double-stranded template DNA, the random primer was annealed and extended by using T4 DNA polymerase at a low temperature (Fig. 1a and b). Extended products are subsequently amplified by PCR with a primer comprising only the constant part of the random primer. This step probably involves an overlap extension PCR, as outlined in Fig. 1c.

Sequence-independent amplification of template DNA of high complexity should lead to a homogeneous size distribution of the PCR products appearing as a smear after size separation by agarose gel electrophoresis. The use of 1 ng of human genomic DNA as a template for the random PCR indeed resulted in a smear ranging from ca. 180 bp to 1 kb (Fig. 2, lane a). Since the amount of nucleic acids that can be extracted from virus particles is limited, we determined the amount of DNA required to generate detectable PCR products after random PCR. A homogeneous smear was seen at amounts of template DNA down to 100 pg. With decreasing amounts of template DNA, the homogeneous smear was more and more substituted by the appearance of distinct bands. Random PCR with 100 fg of template DNA resulted in only few distinct bands, and no products were seen when water had been used instead of template DNA (Fig. 2). Although distinct bands became dominant with decreasing amounts of template DNA, 1 pg of DNA seemed to result in sufficient amounts of PCR products for further characterization.

Selective isolation of viral nucleic acids. Selective isolation of viral nucleic acids is critical for the specificity of the PAN-PCR approach. To evaluate whether this is achievable at all, lysates of Ad-infected cells containing a complex mixture of cellular nucleic acids, proteins, and Ad5 particles were sepa-

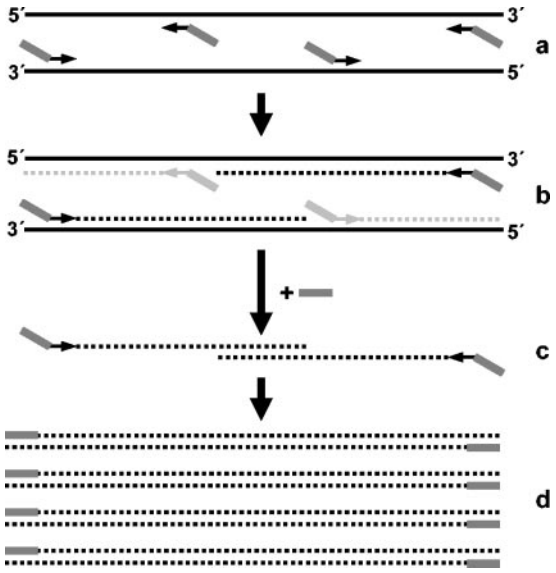

FIG. 1. Proposed mechanism of random-PCR (schematic). (a) Hybridization of the randomized part of the K-random-s primer to template-DNA; (b) primer extension with T4 polymerase; (c and d) PCR with primer K-s: Hybridization and extension of strands generated in panel b (c) and PCR amplification with K-s (d). Continuous line, template-DNA; dotted line, newly synthesized DNA-strand; small arrow, randomized part of K-random-s primer; shaded bar, constant part of K-random-s.

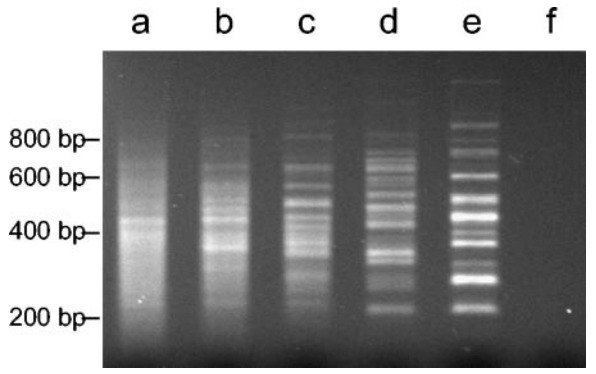

FIG. 2. Sensitivity cutoff of random PCR. Different amounts of template DNA (human genomic DNA digested with DdeI) were used for random amplification as follows: lane a, 1 ng; lane b, 100 pg; lane c, 10 pg; lane d, 1 pg; lane e, 100 fg; lane f, no template DNA.

rated according to density by a two-step CsCl ultracentrifugation approach. DNA was extracted from the banded Ad particles. A total of 1 ng of isolated DNA was amplified by random PCR, resulting in a homogeneous smear similar to results obtained with human genomic DNA. The PCR products were cloned, and lysates of single *Escherichia coli* colonies were PCR amplified with primers spanning the cloning site. Distinct bands differing in size were obtained, and nine such PCR products were randomly chosen for sequencing. Analysis by BLAST search was done to identify the origins of sequences. One clone carried a human sequence. Two sequences resulted from cloned primer dimers. The remaining six clones carried different sequences from Ad5 that were homologous to six different loci spread over the Ad5 genome, with identity scores ranging from 97 to 100%.

The purification of viruses by CsCl density centrifugation requires large amounts of viruses and is also restricted to virus particles with high density. We therefore established a purification strategy for viral nucleic acids applicable for a wide range of different viruses. Enrichment for virus particles is achieved by pelleting filtered, virus-containing supernatants through a 30% sucrose cushion. Since viral nucleic acids within particles are protected from nuclease digestion (1), DNase treatment of pelleted particles was used to further select for viral nucleic acids. The feasibility of this approach for DNA and RNA viruses was tested by using murine Ad1 and an enterovirus isolate identified as Cox-B3 by immunological methods. The copy numbers of viral genomes (virus associated and free nucleic acids) of both supernatants were determined by real-time PCR. For both enterovirus and Ad culture, we found titers of $\sim 10^9$ genomic copies per ml of supernatant.

Processing of 11 ml of supernatants from infected cultures and purification of nucleic acids were done as described above. RNA extracted from enterovirus cultures was reverse transcribed into double-stranded cDNA. DNA prepared from Ad-infected cultures and cDNA from enterovirus cultures was used for random PCR. Agarose gel electrophoresis of products from murine Ad culture showed a smear carrying two prominent bands with sizes of about 450 and 620 bp. Random PCR products from enterovirus virus culture showed a homogeneous size distribution ranging from 300 bp to about 1 kb. After cloning and PCR reamplification of inserts, products with sizes of >260 bp were chosen for sequencing and subsequent determination of homologies to known sequences by BLAST search.

A total of six sequences from supernatant of MAV-1 isolate showed the following homologies. One clone carried a human genomic sequence. The remaining five clones carried three different sequences showing identities of 99% to MAV-1. Two sequences were represented twice, probably due to two independent cloning events of a prominent PCR product.

A total of 12 clones derived from supernatant of cell cultures infected with an isolate previously identified as Cox-B3 were chosen for sequencing and analyzed by BLAST search. For one sequence no significant homology could be found in a nucleotide-nucleotide BLAST search. Even a translated BLAST search could not elucidate its origin. Two clones carried human sequences. The remaining nine clones carried sequences that showed homologies to four different regions of enterovirus genome. Two sequences showed highest identity scores for Cox-B3 (81 to 86%, GenBank accession number AF231763), whereas more 3′ sequences had higher similarity to echovirus 14 (88 to 93%; GenBank accession number AY302540). Sequencing of these clones provided evidence for recombination events. Two identical clones carried a PCR product that showed recombination of enterovirus and *Mycoplasma* sequences. One clone carried a sequence that seems to have been generated from recombination of two different loci within the enterovirus genome.

Identification of a patient-derived unknown isolate. As a first application of the PAN-PCR approach, we used an isolate from mouth-washing material of a patient with chronic fatigue

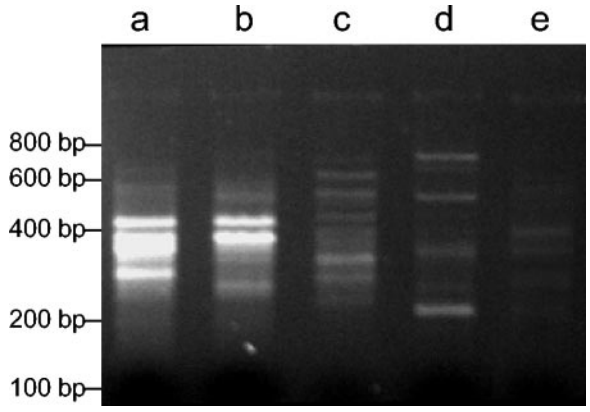

FIG. 3. PAN-PCR products from different templates originating from the virus isolate of a patient with chronic fatigue syndrome. Lanes: a, purified DNA; b, purified DNA digested with DdeI; c, double-stranded cDNA from purified RNA; d, double-stranded cDNA from purified RNA digested with DdeI; e, no template control.

syndrome and high antibody titers to Cox-B2. HeLa cells showed a clear CPE after inoculation with the mouth-washing material. Because of the patient's antibody titers to Cox-B2 and because the isolate had been obtained from material from the respiratory tract, the isolate had been tested for enteroviruses and respiratory viruses such as influenza viruses A and B, parainfluenza viruses 1 to 3, respiratory syncytial virus, and Ad by immunofluorescence and PCR, but none of these tests showed a positive result. To characterize the virus isolate by random PCR, virus particles were concentrated and purified by ultracentrifugation of 10 ml of supernatant of this culture. Instead of pelleting the virus particles through a single sucrose cushion, a sucrose step gradient (20 to 60% in 10% steps) was used. Fractions obtained from the interphase of the step gradient were tested for infectivity by inoculating HeLa cells and monitoring them for the occurrence of CPE. A strong CPE was seen in the fraction taken from the interphase between 30 and 40% sucrose, whereas fractions of lower and higher density showed no or only minor CPE.

The fraction showing the strongest CPE (40%) was further processed for PAN-PCR. Products derived from undigested isolated DNA showed a smear ranging from about 180 to 700 bp with few prominent bands between 300 and 600 bp (Fig. 3, lane a). Since fragmentation of high-molecular-weight genomic DNA by restriction digestion had resulted in a more homogeneous smear after random PCR, the isolated DNA was also digested with DdeI. However, this did not seem to increase the amount of different PCR products generated (Fig. 3, lane b). PCR products derived from double-stranded cDNA were much weaker, showing almost no smear and few distinct bands only (Fig. 3, lanes c and d). Some faint bands were visible when water was added instead of template DNA (Fig. 3, lane e).

PCR products from digested and undigested DNA and from the undigested cDNA were cloned and sequenced. From 14 RNA-derived cloned PCR products, 11 different sequences were obtained, 3 of which showed no homology to any sequence deposited in GenBank. A small subset of clones carried sequences with human origin, mostly from 23S RNA, whereas few single sequences with homologies to *Mycoplasma* sp., *Streptomyces* sp., and *E. coli* were found in the remaining clones. None of the RNA-derived clones had inserts with even low sequence homologies to known viruses. In contrast, 24 of 25 of the DNA-derived cloned PCR products showed homology to HSV-1. A total of 16 different HSV-1 sequences, spread over the entire genome, were obtained. Homology scores ranged from 97 to 100%. To confirm these findings, a quantitative real-time PCR specific for HSV-1 was used to determine genome copy numbers in the DNA preparations from the crude supernatant of the infected culture and from purified viral particles. The crude supernatant contained ca. 6×10^9 HSV-1 genome copies per ml, whereas nucleic acids extracted from DNase-treated virus particles purified from 10 ml of supernatant contained 2×10^8 genome copies. Furthermore, upon retrospective analysis, the original mouth-washing material also tested positive for HSV-1-DNA by PCR.

DISCUSSION

Although the identification of HSV-1 in mouth-washing material is not surprising in retrospect, it clearly demonstrates the applicability of the PAN-PCR approach. The success of this approach critically depends on preferential isolation of viral nucleic acids over a vast excess of cellular nucleic acids. This was achieved by exploiting three properties shared by most, if not all viruses. Due to their small size, viruses can be filtered through a 0.22-μm-pore-size filter. The density of virus particles usually exceeds 1.11 g/ml, allowing purification of virus particles by ultracentrifugation through a 30% sucrose cushion. In addition, viral nucleic acids within particles are protected from digestion with nucleases. Due to RNA instabilities and difficulties in the reliable inactivation of RNases, we only used DNase digestion in our purification protocol. Since the amounts of viral nucleic acids that can be purified from the virus particles are usually limited, we used a novel random PCR to generate sufficient amounts of DNA for cloning and sequencing. Although some sequences are probably more efficiently amplified by random PCR than others, the complexity of the PCR products was sufficient to detect viral sequences present in the extracted nucleic acids. Detection of up to 16 different sequences from one viral genome further suggests that most viral genomes should have sequences that can be efficiently amplified by this PAN-PCR.

One limitation of the random PCR shared by other molecular biological methods for identification of new viruses is that sequences that do not show any homology at all to known viruses cannot be classified. Obtaining more different sequences from the same virus isolate therefore enhances the chances that sequences showing at least a low degree of conservation within a virus family are obtained.

Other molecular biological techniques have been used to identify partial sequences of unknown virus isolates. The initial sequences of SARS coronavirus were discovered by low-stringency PCR with primers targeting highly conserved regions of viral genomes (4), whereas the human metapneumovirus and a novel coronavirus were identified by differential display based methods (13, 14). Allander et al. (1) described a virus discovery method based on ligation-mediated PCR. Although they also use DNase resistance to select for viral nucleic acids, our pro-

tocol includes an additional selection based on particle density, which should increase the specificity for viral nucleic acids. In contrast to ligation-mediated PCR, a random PCR was used in the present study, which is similar to the DOP-PCR previously described for amplification of microdissected chromosomes (2, 15). Pilot experiments suggested that the modified primer sequences and reaction conditions increase amplification efficiency and sensitivity. Thus, the PAN-PCR approach described here should provide an effective alternative that can be performed with small amounts of isolated virus. Obtaining several sequences from different regions of the viral genome from a single random PCR might also be advantageous. Therefore, the PAN-PCR approach should be a useful tool for characterization of virus isolates that have tested negative for all expected viruses.

ACKNOWLEDGMENTS

We thank Regina Bütermann, Rosi Bohr, Klaus Sure, Heike Seidenstücker, and Vera Siegmund for excellent technical assistance.

This study was supported by the FoRUM Forschungsfoerderung of the Ruhr University of Bochum.

REFERENCES

1. **Allander, T., S. U. Emerson, R. E. Engle, R. H. Purcell, and J. Bukh.** 2001. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. Proc. Natl. Acad. Sci. USA **98:**11609–11614.
2. **Bohlander, S. K., R. Espinosa III, M. M. Le Beau, J. D. Rowley, and M. O. Diaz.** 1992. A method for the rapid sequence-independent amplification of microdissected chromosomal material. Genomics **13:**1322–1324.
3. **Burrows, J., A. Nitsche, B. Bayly, E. Walker, G. Higgins, and T. Kok.** 2002. Detection and subtyping of Herpes simplex virus in clinical samples by LightCycler PCR, enzyme immunoassay, and cell culture. BMC Microbiol. **2:**12.
4. **Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz, and H. W. Doerr.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1967–1976.
5. **Fouchier, R. A., N. G. Hartwig, T. M. Bestebroer, B. Niemeyer, J. C. de Jong, J. H. Simon, and A. D. Osterhaus.** 2004. A previously undescribed coronavirus associated with respiratory disease in humans. Proc. Natl. Acad. Sci. USA **101:**6212–6216.
6. **Graham, F. L., and L. Prevec.** 1995. Methods for construction of adenovirus vectors. Mol. Biotechnol. **3:**207–220.
7. **Heim, A., C. Ebnet, G. Harste, and P. Pring-Akerblom.** 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J. Med. Virol. **70:**228–239.
8. **Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand.** 1991. Detection of specific polymerase chain reaction product by utilizing the 5′ → 3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. **88:**7276–7280.
9. **Marre, R., T. Mertens, M. Trautmann, and E. Vanek.** 2000. Klinische Infektiologie. Urban & Fischer Verlag, Munich, Germany.
10. **Rohde, G., A. Wiethege, I. Borg, M. Kauth, T. T. Bauer, A. Gillissen, A. Bufe, and G. Schultze-Werninghaus.** 2003. Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalisation: a case-control study. Thorax **58:**37–42.
11. **Rose, T. M., J. G. Henikoff, and S. Henikoff.** 2003. CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. Nucleic Acids Res. **31:**3763–3766.
12. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **van den Hoogen, B. G., J. C. de Jong, J. Groen, T. Kuiken, R. de Groot, R. A. Fouchier, and A. D. Osterhaus.** 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat. Med. **7:**719–724.
14. **van der Hoek, L., K. Pyrc, M. F. Jebbink, W. Vermeulen-Oost, R. J. M. Berkhout, K. C. Wolthers, P. M. E. Wertheim-van Dillen, J. Kaandorp, J. Spaargaren, and B. Berkhout.** 2004. Identification of a new human coronavirus. Nat. Med. **10:**368–373.
15. **von Eggeling, F., and H. Spielvogel.** 1995. Applications of random PCR. Cell Mol. Biol. **41:**653–670.
16. **Wang, D., A. Urisman, Y. T. Liu, M. Springer, T. G. Ksiazek, D. D. Erdman, E. R. Mardis, M. Hickenbotham, V. Magrini, J. Eldred, J. P. Latreille, R. K. Wilson, D. Ganem, and J. L. DeRisi.** 2003. Viral discovery and sequence recovery using DNA microarrays. PLoS Biol. **1:**257–260.